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(54) Title: GENE THERAPY METHOD USING FGF-5

(57) Abstract

A method is described for introducing an FGF-5 nucleic acid sequence into a mammalian host cell. The FGF-5 nucleic acid sequence lacks the signal sequence so that cells that are transformed with the sequence will not become tumorigenic. It is intended that the FGF-5 sequence is introduced into mammalian cells to promote angiogenesis. Preferably, the FGF-5 sequence is introduced into a human patient to treat myocardial ischemia or peripheral vascular disease.

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GENE THERAPY METHOD USING FGF-5

Field of the Invention

The present invention is in the field of gene therapy. More specifically, the present
5 invention is in the field of gene therapy using the FGF-5 gene.

Background of the Invention

Fibroblast growth factors (FGFs) comprise a family of proteins with related amino acid structure. They are encoded by distinct genes and share sequence homology. Even
10 though there are more than five FGFs, FGFs 1-5 will be discussed here. For example, FGF-1 is acidic FGF, FGF-2 is basic FGF, FGF-3 is int-2, FGF-4 is KFGF or HST, and FGF-5 is described herein.

The FGF-5 of the present invention was originally isolated as an oncogene. See
Goldfarb *et al.* U.S. Patent Nos. 5,155,217 and 5,238,916, Zhan *et al.* "Human Oncogene
15 Detected by a Defined Medium Culture Assay" (*Oncogene* (1987) 1:369-376), Zhan *et al.*
"The Human FGF-5 Oncogene Encodes a Novel Protein Related to Fibroblastic Growth
Factors" (*Molecular and Cellular Biology* (1988) 8:3487-3495), and Bates *et al.*
"Biosynthesis of Human Fibroblast Growth Factor 5" (*Molecular and Cellular Biology*,
(1991) 11:1840-1845). The disclosure of each of these patents and articles is hereby
20 incorporated by reference in their entireties. The FGF-5 oncogene nucleic acid sequence
was reported in both Goldfarb patents and in the Zahn *et al.* article (1988, 8). As
discussed in each of these references, the FGF-5 gene is an oncogene which can transform
cells into a tumorigenic state. Additionally, reports in the literature show that the related
genes int-2 and HST can transform cells to be tumorigenic. See Theillet *et al.*, *Oncogene*
25 (1989) 4:915-922, and Goldfarb *et al.*, *Oncogene* (1991) 6:65-71.

Consequently, it is the aim of the present invention to use the FGF gene in gene therapy with human patients, while removing the oncogenic potential of this gene.

Summary of the Invention

The present invention relates to a method for expressing FGF-5 *in vivo*, comprising introducing a nucleic acid sequence encoding FGF-5, without a signal sequence, into a vector that can infect mammalian cells and cause these cells to express FGF-5 without causing the cells to become tumorigenic.

5 The present inventor has discovered that to use FGF-5 in a gene therapy model in human patients, one must remove the signal sequence before administering the gene. Otherwise, the gene therapy may transform normal human cells into tumorigenic cells, which is obviously undesirable.

10 More specifically, the present invention relates to a gene therapy method for introducing an FGF-5 gene into a human cell of a patient suffering from myocardial ischemia or peripheral vascular disease comprising:

constructing a retroviral vector having a nucleic acid sequence encoding FGF-5, without a signal sequence, having an N terminus of GGGAGAAGCG TCTCGCCCCC
15 AAAG (SEQ ID NO: 1), in operable linkage with the appropriate regulatory elements necessary to express the FGF-5 nucleic acid sequence in a human cell, to form the FGF-5 protein; and

introducing the vector into a cellular area in the human patient which is in need of treatment with the FGF-5 protein.

20

Brief Description of the Drawings

Figure 1-A and Figure 1-B are the nucleic acid sequence for the FGF-5 gene which includes the signal sequence.

25 Figure 2 is the amino acid sequence for the FGF-5 gene which includes the signal sequence.

Figure 3 is the nucleic acid sequence for the FGF-5 gene starting at the 22nd amino acid of the sequence of Figure 1.

Figure 4 is the amino acid sequence for the FGF-5 gene starting at the 22nd amino acid of the sequence of Figure 2.

Detailed Description of the Invention

As shown in Goldfarb *et al.* (U.S. Patent No. 5,155,217, the disclosure of which is hereby incorporated by reference in its entirety), FGFs 1-5 share a sequence homology between 41 and 50%. For example, column 9 of Goldfarb ('217) shows that there is 45% sequence identity between FGF-5 and basic FGF, 41% sequence homology between FGF-5 and acidic FGF, 52% sequence homology between FGF-5 and KFGF (also called HST), and 50% sequence homology between FGF-5 and int-2 (Goldfarb has used the designation FGF-3 throughout '217 but later changed the identity of their protein to FGF-5). See also Goldfarb *et al.* U.S. Patent No. 5,238,916. Basic FGF is more fully in U.S. Patent No. 5,155,214; 4,994,559; 5,401,701; and 5,439,818. Acidic FGF is disclosed in U.S. Patent No. 5,312,911. The disclosures of all of the U.S. patents listed above are hereby incorporated by reference in their entireties.

The FGF-5 protein has been shown to be synthesized *in vitro* in animal cells to yield a 29,500-dalton protein which was secreted from tumor cells as a glycoprotein containing heterogeneous amounts of sialic acid. Glycosidase treatment suggested that FGF-5 has both N-linked and O-linked sugars. See Bates *et al.* "Biosynthesis of Human Fibroblast Growth Factor 5" (*Molecular and Cellular Biology*, (1991) 11:1840-1845), hereby incorporated by reference in its entirety.

The present invention describes the use of the FGF-5 nucleic acid sequence in a gene therapy method whereby the FGF-5 sequence is converted from an oncogene to a protooncogene (non tumorigenic) before it is introduced into human cells. As described above, the gene sequences are disclosed in the two Goldfarb patents ('217 and '916) and Zahn *et al.* "The Human FGF-5 Oncogene Encodes a Novel Protein Related to Fibroblastic Growth Factors" (*Molecular and Cellular Biology* (1988) 8:3487-3495), which are all hereby incorporated by reference in their entireties.

The FGF-5 oncogene is a 267 amino acid protein as compared to int-2, which is 240, HSTKS3, which is 206, and acidic and basic FGFs which are both 155 amino acids long. See Figures 1 and 2 for the nucleic acid and amino acid sequences of FGF-5, including the signal sequence. As stated above, the signal sequence of the FGF-5 oncogene must be removed before incorporating it into a gene therapy vector for human

use. It is acceptable if enough of the signal sequence is removed so that the tumorigenic properties are eliminated from the FGF-5 molecule described in the Golfarb patent. Preferably, between 10 and 30 amino acids are removed from the N-terminus. More preferably, between 15 and 25 amino acids are removed from the N-terminus. Most 5 preferably, the first 22 amino acids are removed from the N-terminus. (See Clements *et al.*, *Oncogene* (1993) 8:1311-1316 which is hereby incorporated by reference in its entirety). See Figures 3 and 4 for the FGF-5 nucleic acid and amino acid sequences which begin at the 22nd amino acid of the sequences shown in Figures 1 and 2. Clements *et al.* disclose prokaryotic expression of the mature form of FGF-5 and describe silent mutations 10 in the 5' end of the cDNA insert that increase the expression levels of FGF-5. The FGF-5 molecule of the present invention preferably contains those mutations.

The present gene therapy method of delivering FGF-5 to local areas in human patients is useful to treat human diseases of the vascular system, as well as enhancing the ability of neural cells to proliferate and for bone growth. See Morrison *et al.* "Basic 15 Fibroblast Growth Factor supports the survival of cerebral cortical neurons and primary culture" *Proc. Natl. Acad. Sci. (USA)* (1986) 83:7537-7541, which are hereby incorporated by reference in their entireties. There is also evidence that FGF-5 is a major muscle-derived survival factor for cultured spinal motor neurons (Hughes *et al.*, *Neuron* (1993) 10:369-367), that FGF-5 is present in adult mouse central nervous system (Haub *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1990) 87:8022-8026), that FGF-5 is a regulator of the hair 20 growth cycle (Hebert *et al.*, *Cell* (1984) 78:1017-1025), that FGF-5 promotes differentiation of cultured rat neurons (Lindholm *et al.*, *European Journal of Neuroscience* (1994) 6:244-252), that FGF-5 may play a role in limbic system function or dysfunction (Gomez-Pinilla *et al.*, *Brain Research* (1993) 606:79-86), that FGF-5 can play a role in the 25 biology of the outer retina (Bost *et al.*, *Exp. I. Res.* (1992) 55:727-34), that basic FGF can ameliorate learning deficits in basal forebrain-lesioned mice (Ishihara *et al.*, *Jpn. J. Pharmacol.* (1992) 59:7-13). Fibroblasts that have been engineered to express bFGF without a signal sequences have a more robust effect on the viability and function of grafted dopaminergic neurons than with fibroblasts that express bFGF with a signal 30 sequence (see Takayama *et al.*, *Nature Medicine* (1995) 1:53-58). bFGF appears to be

neuroprotective and neurotrophic (see Cheng and Mattson, *Neuron* (1991) 7:1031-1041; Freese *et al.*, *Brain Research* (1992) 575:351-355; Finkelstein *et al.*, *Stroke* (1993) 24 (supp. 1):141-143) angiogenic (Baffour *et al.*, *Jour. Vasc. Surg.* (1992) 16:181-191); and osteogenic (Kawaguchi *et al.*, *Endocrinology* (1993) 135:774-781; Nagai *et al.*, *Bone* 5 (1995) 16:367-373; Nakamura *et al.*, *Endocrinology* (1995) 136:1276-1284; and Mayahara *et al.*, *Growth Factors* (1993) 9:73-80). Also, it is contemplated that the FGF-5 gene will be useful for many of the uses shown for other FGFs. Accordingly, delivery of the FGF-5 gene will be useful in a variety of vascular, cardiovascular, neuronal, osteogenic, and other indications to correct or regulate cellular dysfunction. Preferably, the FGF-5 gene 10 administered for angiogenic uses or to support their growth and/or proliferation or neuronal cells. More preferably, the FGF-5 nucleic acid sequence is administered to promote blood vessel growth in myocardial ischemia.

Definitions

15 The term "polynucleotide" or "nucleic acid sequence" as used herein refers to a polymer of nucleotides of any length, preferably deoxyribonucleotides, and is used interchangeably herein with the terms "oligonucleotide" and "oligomer." The term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, as well as antisense polynucleotides. It also includes known types 20 of modifications, for example, the presence of labels which are known in the art, methylation, end "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, replacement with certain types of uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) or charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), introduction of pendant moieties, such as, for example, 25 proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive species, boron, oxidative moieties, etc.), alkylators (e.g., alpha anomeric nucleic acids, etc.). The term "gene" is used to describe the coding sequence for the polypeptide of interest, 30 for example, FGF-5.

By "genomic" is meant a collection or library of DNA molecules which correspond to the sequence found in chromosomal DNA as opposed to spliced mRNA. By "cDNA" is meant a DNA sequence that hybridizes to a complimentary strand of mRNA.

5 "Regulatory" or "control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in eukaryotes, generally, such control sequences include promoters and transcription termination sequences. The term "control sequences" is intended to include, at a
10 minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

15 "Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence so that expression of the coding sequence is achieved under conditions compatible with the control sequences.

20 A "vector" or "plasmid" is a nucleic acid sequence in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment in a host cell. Vectors are used routinely in recombinant DNA techniques. Any extrachromosomal small genome such as a plasmid, phage, or virus is a potential vector.

25 "Retroviral vector" is a vector derived from a retrovirus and it has the capability to insert a gene or DNA fragment into the host chromosomal genome by a recombinational event, so that the DNA fragment can be expressed in a host cell. See Singer, M. and Berg, P., *Genes and Genomes*, Mill Valley, CA (1991) pp. 310-314, which is hereby incorporated by reference. Retroviruses are RNA viruses (the viral genome is RNA). The genomic RNA is reverse transcribed into DNA after it enters the cell and then it is integrated stably and efficiently into the chromosomal DNA of
30 transduced cells. See Mulligan, R.C., In: *Experimental Manipulation of Gene*

Expression, M. Inouye (ed), 155-173 (1983); Mann, R. *et al.*, *Cell* (1983) 33:153-159; Cone, R.D. and R. C. Mulligan, *Proc. Natl. Acad. Sci. (USA)*, (1984) 81:6349-6353 which are hereby incorporated by reference in their entireties.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, particle mediated, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. Examples of particle mediated transduction are shown in U.S. Patent Nos. 4,945,050 and 5,149,655, which are hereby incorporated by reference in their entireties.

"Homology" refers to the degree of similarity between x and y. The correspondence between the sequence from one form to another can be determined by techniques known in the art. For example, they can be determined by a direct comparison of the sequence information of the polynucleotide. Alternatively, homology can be determined by hybridization of the polynucleotides under conditions which form stable duplexes between homologous regions (for example, those which would be used prior to S₁ digestion), followed by digestion with single-stranded specific nuclease(s), followed by size determination of the digested fragments.

As used herein, x is "heterologous" with respect to y if x is not naturally associated with y in the identical manner; i.e., x is not associated with y in nature or x is not associated with y in the same manner as is found in nature.

As used herein, the term "protein" or "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, polypeptides, proteins, and polyproteins, as well as fragments of these, are included within this definition. This term also does not refer to, or exclude, post expression modifications of the protein, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, proteins containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.).

proteins with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

- A polypeptide or protein or amino acid sequence "derived from" or "coded by" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.
- "Alleles" and "variants" refers to a polypeptide that differs from the native specified protein by virtue of one or more amino acid substitutions, deletions, or insertions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acid residues such as to alter a glycosylation site, a phosphorylation site, an acetylation site, or to alter the folding pattern by altering the position of the cysteine residue that is not necessary for function, etc. Conservative amino acid substitutions are those that preserve the general charge, hydrophobicity/hydrophilicity and/or steric bulk of the amino acid substituted, for example, substitutions between the members of the following groups are conservative substitutions: Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, and Phe/Trp/Tyr.
- "Signal sequence" is used to describe the N-Terminal amino acids that enable the polypeptide to be transported outside the boundaries of the cells in which it is made. As stated above, it is this sequence that enables the FGF-5 nucleic acid sequence to transform cells into a tumorigenic state. In FGF-5, it is the first 59 or, more preferably, the first 61 amino acids at the N-Terminus that constitute the signal sequence.
- The term "cardiovascular indication" as used herein refers to a diagnosis or presumptive diagnosis of cardiovascular disease or conditions affecting the heart that are associated with atherosclerosis, ischemic syndromes, cardiomyopathies, arrhythmias, dysrhythmias, hypertension and infections. The diagnosis can be made based on pain, fatigability, weakness, palpitations, and systemic symptoms that may be due to the cardiac disease or that may accompany it. Determination of a cardiovascular indication

may include a physical exam and other non-invasive diagnostic procedures including radionuclide imaging, positon emission tomography, magnetic resonance imaging, echocardiography, and can also include venous and arterial cannulation and pulmonary and cardiac catheterization used in diagnosis of the cardiac condition.

5 The term "administering to intrapericardially" or "administering into the pericardial space" as used herein refers to any method of administration that effects delivery of a therapeutic agent into the pericardial space. The pericardial space may be the entire region comprising the pericardial space, or only a part of it. The term "administering into pericardial space" is synonymous with the terms "intrapericardial delivery" and "pericardial delivery", and can include delivery to subregions of the pericardial space that form interfaces between the pericardial space and the tissue that surrounds and forms it. The administration into pericardial space can be accomplished by, for example, the following means of administration including injection, laser, catheter, pump. Intrapericardial delivery of the polynucleotides and the drugs of the
10 invention can be accomplished by the methods of such delivery as disclosed in, for example, U.S. Patent Nos. 5,137,510, 5,269,326, and 5,213,570, herein incorporated by reference.
15

Vectors and Expression Systems

20 The following expression systems describe vectors, promoters and regulatory elements that are useful for gene therapy applications for the delivery of the FGF-5 polynucleotide. Vectors and expression systems useful for the present invention include viral and non-viral systems. Example viral delivery systems include retroviruses, adenoviruses, adeno-associated viruses (AAV),
25 sindbis and herpes viruses. In one aspect of the present invention, the viral vector is capable of integrating the FGF-5 nucleic acid sequence into the host cell genome for long term expression. Examples of vectors that can integrate in this fashion are retroviruses and AAV. One preferred retrovirus is a murine leukemia virus. However, it may be preferred to avoid integration into the host
30 cell genome. For example, when short term administration of FGF-5 is

required, long term expression can be unnecessary and possibly undesirable.

Non-viral vectors include naked DNA and DNA formulated with cationic lipids or liposomes.

Preferably, the FGF-5 nucleic acid coding sequence is administered in
5 one of the above systems to a patient's cells without the signal sequence. The description below is directed to means for including the FGF-5 coding sequence in a larger sequence that will facilitate expression of the FGF-5 polypeptide.

Retroviral vectors are produced by genetically manipulating retroviruses.
Retroviral vectors are effective for integration into the host cell genome, as
10 explained above. However, they only infect dividing cells. Retroviral vectors contain RNA. In the present invention the viral RNA vector contains the FGF-5 gene, and once it enters the cell, it is reverse transcribed into DNA and stably integrated into the host cell genome.

The wild type retrovirus genome contains three genes: the gag, pol, and
15 env genes, which are flanked by the long terminal repeat (LTR) sequences. The gag gene encodes the nucleocapsid proteins, the pol gene encodes the viral enzymes including reverse transcriptase and integrase, the env gene encodes the viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of virion RNAs. Adjacent to the 5' LTR are sequences
20 necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsulation of viral RNA into particles (the Psi site). See Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R. et al., *Cell* (1983) 33:153-159; Cone, R.D. and R. C. Mulligan, *Proc. Natl. Acad. Sci. (USA)*, (1984) 81:6349-6353.

25 More specifically, the present invention contemplates constructing a vector in which the gag, pol, and env genes are removed and replaced with the FGF-5 gene. The LTR, psi sequence and primer binding sites are also present to facilitate vector replication. The vector is transformed into a packaging animal cell line which contains the gene sequences for the gag, pol, and env genes in its
30 genome and which constitutively express those proteins. These proteins are

usually expressed from a heterologous promoter (eg. CMV) and the genes are not operably linked to sequences (such as psi, LTR which are required for viral replication). This cell will make empty viral particles and is a recipient for the vector described above which contains the FGF-5 gene, the psi and primer binding sequences as well as the LTR sequences. The cell can be transiently transfected with the vector to produce the product (viral particle with the FGF-5 vector). Preferably, the product virions are used to infect a second packaging cell line which then can permanently produces the viral particles.

The retroviral vector can be packaged by transfecting the FGF-5 nucleic acid sequence into cells expressing the gag-pol and env genes. These "packaging cell lines" are mammalian tissue culture cell lines which express structural proteins of a retrovirus and produce retrovirus-like particles. They are incapable of producing infectious virions. Transfected retroviral vectors (with the FGF-5 nucleic acid sequence) into packaging cell lines results in the production of retroviral vector particles with the desired genetic construction. Packaging cell lines are publically available and include Crip, GPE86, PA317, and PG13. See Miller *et al.*, *J. Virol.* (1991) 65:2220-2224, Cone *et al.*, *Proc. Natl. Acad. Sci. (USA)*, (1988) 85:6460-6464, Eglitis *et al.*, *Biotechniques* (1988) 4:608-614, Miller *et al.*, *Human Gene Ther.* (1990) 1:5-14, which are all hereby incorporated by reference in their entireties.

Also, AAV are advantageous because they replicate to a high titer, they integrate efficiently, are not pathogenic to humans, are stable, easy to purify, and they infect non-dividing cells. An AAV vector is constructed by inserting the FGF-5 coding sequence, under the control of a suitable promoter/enhancer, between the AAV LTRs, which are the only sequences required in cis for AAV replication. This DNA construct is transfected into a suitable human cell line in the presence of another plasmid which expresses Rep and CAP, the AAV coding regions needed for replication. At a suitable time post-transfection, the cells are infected with a helper virus, such as Adenovirus or Herpes Simplex virus. After infection, vector particles carrying the FGF-5 gene are harvested from these

cells. The AAV particles are purified from contaminating Adenovirus or Herpes Virus by standard protocols.

Adeno virus is advantageous because it infects a wide variety of cells, infects non-dividing cells, produces a high titer, the biology is well understood, 5 and it can accept large inserts. The adenovirus gene expression is controlled by a cascade of genes. For example, the gene expression order is "immediate early", "early", DNA synthesis, and late or structural genes. These genes are turned on in sequence. The master gene that is turned on first is E1A. One preferred embodiment would involve replacing the E1A gene with the FGF-5 10 gene and transfecting this vector into cells that constitutively produce E1A, such as 293 cells which are publically available. The vector contains all the genes necessary for virion production and the cell line provides the missing E1A protein. Consequently, the virion is produced which contains the FGF-5 sequence.

15 One non-viral system that can be used is the T7/T7 system. Here a short promoter sequence recognized by the bacterial virus T7 polymerase is placed on a vector upstream of the FGF-5 gene. The vector can then be inserted into cells and the missing T7 polymerase can be added to obtain gene transcription. Alternatively, a vector containing the following sequences can be made, the T7 20 promoter sequence, the T7 polymerase gene, another copy of the T7 promoter sequence, and the FGF-5 gene. In this embodiment, the vector is transformed into cells and simply requires a small amount of T7 polymerase to initiate. Therafter, the vector directs the manufacture of its own polymerase.

Although the methodology described is believed to contain sufficient 25 details to enable one skilled in the art to practice the present invention, other items not specifically exemplified, such as plasmids, can be constructed and purified using standard recombinant DNA techniques described in, for example, Sambrook *et al.* (1989), MOLECULAR CLONING: A LABORATORY MANUAL, 2d edition (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.), 30 and Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY

(1994), (Greene Publishing Associates and John Wiley & Sons, New York, N.Y.). under the current regulations described in United States Dept. of HEW,

NATIONAL INSTITUTE OF HEALTH (NIH) GUIDELINES FOR

RECOMBINANT DNA RESEARCH. These references include procedures for

5 the following standard methods: cloning procedures with plasmids,

transformation of host cells, cell culture, plasmid DNA purification, phenol

extraction of DNA, ethanol precipitation of DNA, agarose gel electrophoresis,

purification of DNA fragments from agarose gels, and restriction endonuclease

and other DNA-modifying enzyme reactions.

10 Gene therapy can be practiced according to the invention by genes that are under regulatory control of appropriate regulatory sequences for transformation or infection of myocytes, cells within the pericardium, cells at the epicardium, or any cells in a region of the heart accessible to an intrapericardially delivered gene.

When the genes are directed to nerve cells, the genes must be under the

15 appropriate regulatory elements that enable expression in those cells. Gene therapy can be practiced as follows using coding regions for any therapeutic appropriate for treatment of a cardiovascular or neural indication.

As explained above, gene therapy strategies for delivery of the FGF-5 gene nucleic sequence can utilize viral or non-viral vector approaches in *in vivo* or *ex vivo* 20 modality. Expression of such coding sequence can be induced using endogenous mammalian, viral or other heterologous promoters. Expression of the coding sequence *in vivo* can be either constitutive or regulated.

For delivery using viral vectors, any of a number of conventional viral vectors can be used, as described in Jolly, *Cancer Gene Therapy* (1994) 1:51-64. Promoters 25 that are suitable for use with these vectors are also conventional in the art and include the Moloney retroviral LTR, CMV promoter and the mouse albumin promoter. Virus competent for one round of replication can be produced and injected directly into the animal or humans or by transduction of an autologous cell *ex vivo*, followed by injection *in vivo* as described in Zatloukal *et al.*, *Proc. Natl. Acad. Sci. USA* (1994) 91:5148-

30 5152.

Delivery

- Preferably, the FGF-5 gene is administered to the local area of the pericardium or neural cells. More preferably, the FGF-5 gene is delivered to the pericardium without the signal sequence. The FGF-5 nucleic acid sequence may be delivered into the pericardial space by any method conventional in the art, such as that described in Barr *et al.*, *Gene Therapy* (1994) 1:51-58. Barr *et al.* describe gene delivery via catheter-mediated infusion of replication defective adenovirus into the coronary arterial circulation. High level expression of an exogenous gene was obtained throughout the thickness of the ventricular and atrial walls within the distribution of the injected coronary artery. The FGF-5 nucleic acid sequence may be linked to tissue specific promoters or leader sequences for expression in cardiac muscle cells, for example, the untranslated leader sequence of dystrophin DNA, or regulatory regions in the muscle creatine kinase gene such as that described in Cox *et al.*, *Nature* (1993) 364:725-729.
- Delivery of genes to the intrapericardial space is a safer and more effective method of accomplishing myocardial gene therapy. Accordingly, delivery of genes to the pericardial space does not require mechanical violation of the myocardium as does direct myocardial injection. Because intrapericardially delivered agents have access to the entire myocardial surface the ease and effectiveness with which genes can be delivered to large areas of myocardium is increased. Access the coronary circulation causes perfusion of the entire heart with these agents. Also, the pericardium is more easily transducible than myocardium and, thus, that expression of gene products in the pericardial space retains access to myocardium.
- Furthermore, the exposure time of nucleic acids and/or viruses to cells, which is an important determinant of transduction or infection efficiency, increases. Genetic agents deposited in the pericardial space are not subject to rapid dilution, drainage, or dissipation due to blood flow or lymphatic clearance, and thus have much longer exposure times than vascularly delivered agents, also increasing the transduction or infection efficiency of the genes. Such an advantage achieved by the method of the invention, translates into much higher transduction

or infection efficiency with genes and/or viruses in either the myocardium or the pericardium than is achievable in the coronary vessel. Lastly, because pericardium is highly efficient at expressing certain proteins and in some cases is even more efficient than myocardium at this task, the method of the invention is a new and 5 improved method of delivery of genes for gene therapy for treatment of a cardiovascular indication.

- Practice of the invention also includes, for example, delivering the FGF-5 genes into the pericardial space, optionally in combination with cardiovascular therapeutics, in liposomal compositions, including heterovesicular liposomes.
- 10 Delivery in liposomes increases the efficacy of the gene or cardiovascular therapeutics, reduces the dosage requirements and augments the benefits of any cardiovascular therapeutic delivered into the pericardial space.

Additionally, the FGF-5 gene can be delivered to nerve tissue. Actual delivery methods may vary, depending on the sites of the nerves to be affected. For example, 15 administration to nerve tissue may be by encapsulating the FGF nucleotide sequence in a herpes virus which will specifically target nerve cells.

For *in vivo* therapy, the coding sequence can be delivered into the intrapericardial space by direct injection, or into pericardial tissue by delivery such as, for example, those systems described in U.S. Patent Nos. 5,137,510, 5,213,570, and 20 5,269,326. Promoters suitable for use in this manner include endogenous and heterologous promoters such as those described herein. Any promoter appropriate for the expression of the gene selected for the therapy is contemplated by the method of the invention. The coding sequence can be injected in a formulation comprising a buffer that can stabilize the coding sequence and facilitate transduction thereof into cells and/or 25 provide targeting, as described in Zhu *et al.*, *Science* (1993) 261:209-211.

Expression of the FGF-5 coding sequence *in vivo* (by either viral or non-viral vectors) can be regulated by use of regulated gene expression promoters as described in Gossen *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1992) 89:5547-5551. For example, the coding sequence selected for the therapy can be regulated by tetracycline responsive 30 promoters. These promoters can be regulated in a positive or negative fashion by

- treatment with the regulator molecule. Additionally, the FGF-5 gene may be introduced into cells under the control of promoters which are activated using radiotherapy. For example, U.S. Patent No. 5,205,152 entitled "Cloning and Expression of Early Growth Regulatory Protein Genes" shows that the Egr-1 gene is one of the best radiation induced genes and may be activated by exposure to radiation. WO 92/11033 disclosed genetic constructs which comprise an enhancer-promoter region which is responsive to radiation, and at least one structural gene whose expression is controlled by the enhancer-promoter. The U.S. Patent and the PCT application are hereby incorporated by reference in their entireties.
- 5 For non-viral delivery, the FGF-5 coding sequence can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu and Wu, *J. Biol. Chem.* (1987) 10: 262: 4429-4432; insulin, as described in Hucked *et al.*, *Biochem. Pharmacol.* (1990) 40:253-263; galactose, as described in Plank *et al.*, *Bioconjugate Chem.* (1992) 3:533-539; lactose, as described in Midoux *et al.*, *Nucleic Acids Res.* (1993) 21:871-878; or transferrin, as described in Wagner *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1990) 87:3410-3414. Other delivery systems include the use of liposomes to encapsulate DNA comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters, as described in Nabel *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1993) 90:11307-11311, and Philip *et al.*, *Mol. Cell Biol.* (1994) 14:2411-2418. Further non-viral delivery suitable for use includes mechanical delivery systems such as the biostatic approach, as described in Woffendin *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1994) 25: 91(24):11581-11585. Moreover, the coding sequence and the product of expression of such can be delivered through deposition into the pericardial space of photopolymerized hydrogel materials such as Focalgel®. Furthermore, the FGF-5 gene sequence can be inserted into a host cell by direct uptake or particle mediated transduction. The FGF-5 sequence may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. Examples of particle mediated
- 15 20 25 30

transduction are shown in U. S. Patent Nos. 4,945,050 and 5,149,655, which are hereby incorporated by reference in their entireties.

As stated above, naked DNA can be administered to muscle tissue. See Wolff, JA *et al.* entitled Direct gene transfer into mouse muscle *in vivo*, *Science* (1990) 247:1465-1468; Kitsis *et al.*, Hormonal modulation of a gene injected into rat heart *in vivo*, *Proc. Natl. Acad. Sci.* (1991) 88:4138-4142; Lin *et al.*, Expression of recombinant genes in myocardium *in vivo* after direct injection of DNA, *Circulation* (1990) 82:2217-2221; and Buttrick *et al.*, Behavior of genes directly injected into rat heart *in vivo*, *Circ. Res.* (1992) 70:193-198. The above references are hereby incorporated by reference in their entireties.

To practice one aspect of the invention, the diagnosis of a cardiovascular condition is made, and the appropriate dosages are determined on the basis of the diagnosis. The invention is practiced to prevent, reduce or treat a cardiovascular condition.

The method of the invention applies to any cardiovascular indication, for example a diagnosis of: (1) atherosclerosis, and conditions that predispose one to pathological atherosclerotic plaque development in the coronary arteries including lipid/cholesterol deposition, macrophage/inflammatory cell recruitment, plaque rupture, thrombosis, platelet deposition, neointimal proliferation; (2) ischemic syndromes and attendant syndromes, including but not limited to myocardial infarction, stable and unstable angina, coronary artery restenosis following percutaneous transluminal, coronary angioplasty, reperfusion injury; (3) cardiomyopathies, including but not limited to cardiomyopathies caused by ischemic syndromes, cardiotoxins such as alcohol and chemotherapeutic agents like adriamycin, infections, such as viral, cytomegalovirus (CMV), and parasitic (*trypanosoma cruzi*), hypertension, metabolic diseases, (including but not limited to uremia, beriberi, glycogen storage disease), radiation, neuromuscular disease (such as Duchenne's muscular dystrophy), infiltrative diseases (including but not limited to sarcoidosis, hemochromatosis, amyloidosis, Fabry's disease, Hurler's syndrome), trauma, and idiopathic causes; (4) a/dysrhythmias (including but not limited to a/dysrhythmias resulting from the same causes listed above for cardiomyopathies);

(5) infections (including bacterial, viral, fungal, and parasitic causes); (6) cardiac tumors; (7) inflammatory conditions (including but not limited to myocarditis, pericarditis, endocarditis, immune cardiac rejection and conditions resulting from idiopathic, autoimmune, or connective tissue diseases); and (8) hypertension.

- 5 The FGF-5 nucleotide sequence can be administered to the pericardial space and expressed in the heart tissue, including but not limited to, for example, pericardial tissue, myocardial tissue, epicardial tissue, or perivascular tissue. The sequence can be placed in a vector, such as a viral vector, or a plasmid vector. The polynucleotides may be presented into the pericardial space in any formulation commonly known in the art including buffers, 10 excipients, gels, matrices and polymers. Appropriate formulations for the polynucleotides administered intrapericardially in the practice of the invention also include liposomal preparations such as, for example, those disclosed in U.S. Patent No. 5,422,120, WO 95/13796, WO 94/23697, WO 91/14445 and EP 524,968 B1, particularly including the heterovesicular liposomal preparations disclosed in these patents and applications.
- 15 The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

20

Example 1

- The coding sequence for FGF-5, without the signal sequence, is isolated by standard recombinant DNA techniques and placed in a retroviral vector and encapsulated in viral envelope for delivery intrapericardially. The retrovirus is delivered by laparoscopic cannulation or direct injection into the pericardial space of a patient who is suffering from myocardial ischemia or peripheral vascular disease. Alternatively, the 25 coding sequence is placed in a plasmid vector and the vector is likewise delivered into the pericardial space. The coding sequences are linked with appropriate regulatory sequences and are delivered into the pericardial space by laparoscopic cannulation or direct injection. The FGF-5 nucleic acid sequence is expressed by the patient's cells in the

local area of the release and the FGF-5 protein induces the formation of new blood vessels.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may 5 be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

- 20 -

SEQUENCE LISTING

GGGAGAAGCG TCTCGCCCCC AAAG (SEQ ID NO: 1)
TTCTTCAGCC ACCTGATCCT CAGC (SEQ ID NO: 2)
ATCCTCAGCG CCTGGGCTCA CGGG (SEQ ID NO: 3)
5 CGTCTCGCCC CCAAAGGGCA ACCC (SEQ ID NO: 4)
GGGCAACCCG GACCCGCTGC CACT (SEQ ID NO: 5)

CLAIM:

1. A method for expressing FGF-5 *in vivo*, comprising introducing a nucleic acid sequence encoding FGF-5, without a signal sequence, into a vector that can be introduced into mammalian cells to cause these cells to express FGF-5 without causing the cells to become tumorigenic.

2. A method in accordance with claim 1, wherein the FGF-5 sequence is administered to a human patient to induce angiogenesis in that patient.

3. A method in accordance with claim 1, wherein the vector is a retrovirus, an adenovirus, sindbis virus, herpes virus, or an adeno-associated virus.

A method in accordance with claim 2 wherein the FGF-5 vector is used to treat myocardial ischemia or peripheral vascular disease.

4. A vector comprising a promoter operable in a eukaryotic cell, a nucleic acid sequence encoding FGF-5, without a signal sequence, the FGF-5 nucleic acid sequence being in operable linkage with the promoter.

5. A gene therapy method for introducing an FGF-5 gene into a human cell of a patient suffering from myocardial ischemia or peripheral vascular disease comprising:

constructing a retroviral vector having a nucleic acid sequence encoding FGF-5, without a signal sequence, having an N terminus of GGGAGAAGCG TCTCGCCCCC AAAG (SEQ ID NO: 1), in operable linkage with the appropriate regulatory elements necessary to express the FGF-5 nucleic acid sequence in a human cell, to form the FGF-5 protein; and

introducing the vector into a cellular area in the human patient which is in need of treatment with the FGF-5 protein.

6. A method in accordance with claim 1, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of TTCTTCAGCC ACCTGATCCT CAGC (SEQ ID NO: 2).

7. A method in accordance with claim 1, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of ATCCTCAGCG CCTGGGCTCA CGGG (SEQ ID NO: 3).

8. A method in accordance with claim 1, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of GGGAGAAGCG TCTCGCCCCC AAAG (SEQ ID NO: 1).

9. A method in accordance with claim 1, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of CGTCTCGCCC CCAAAGGGCA ACCC (SEQ ID NO: 4).

10. A method in accordance with claim 1, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of GGGCAACCCG GACCCGCTGC CACT (SEQ ID NO: 5).

11. A method in accordance with claim 4, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of TTCTTCAGCC ACCTGATCCT CAGC (SEQ ID NO: 2).

12. A method in accordance with claim 4, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of ATCCTCAGCG CCTGGGCTCA CGGG (SEQ ID NO: 3).

13. A method in accordance with claim 4, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of GGGAGAAGCG TCTCGCCCC AAAG (SEQ ID NO: SEQ ID NO: 1).

14. A method in accordance with claim 4, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of CGTCTCGCCC CCAAAGGGCA ACCC (ID SEQ ID NO: 4).

15. A method in accordance with claim 4, wherein the nucleic acid sequence encoding FGF-5 has an N terminus of GGGCAACCCG GACCCGCTGC CACT (SEQ ID NO: 5).

FIGURE 1

CCTCTCCCT TCTCTTCCCC GAGGCTATGT CCACCCGGTG CGGCAGGGCG GGCCAGAGCA
GAGGCACGCA GCCGCACAGG GGCTACAGAG CCCAGAACCA GCCCTACAAG ATGCACCTAG
GACCCCGCG GCTGGAAGAA TGAGCTTGTCT CTTCTCCTC CTCCCTTTCT TCAGCCACCT
GATCCTCAGC GCCTGGGCTC ACGGGGAGAA GCGTCTGCC CCCAAAGGGC AACCCGGACC
CGCTGCCACT GATAGGAACC CTAGAGGCTC CAGCAGCAGA CAGAGCAGCA GTAGCGCTAT
GTCTTCTCT TCTGCCTCCT CCTCCCCCGC AGCTTCTCTG GGCAGCCAAG GAAGTGGCTT
GGAGCAGAGC AGTTTCCAGT GGAGCCTCGG GGCGCGGACC GGCAGCTCT ACTGCAGAGT
GGGCATCGGT TTCCATCTGC AGATCTACCC GGATGGAAA GTCAATGGAT CCCACGAAGC
CAATATGTTA AGTGTGTTGG AAATATTGTC TGTGTCTCAG GGGATTGTAG GAATACGAGG
AGTTTCAGC AACAAATTT TAGCGATGTC AAAAAAAGGA AAAACTCCATG CAAGTGCCAA
GTTCACAGAT GACTGCAAGT TCAGGGAGCG TTTTCAAGAA AATAGCTATA ATACCTATGC
CTCAGCAATA CATAGAACTG AAAAAACAGG GCGGGAGTGG TATGTTGCC TGAATAAAAG
AGGAAAAGCC AAACGAGGGT GCAGCCCCCG GTTAAACCC CAGCATATCT CTACCCATT
TCTTCCAAGA TTCAAGCAGT CGGAGCAGCC AGAACTTCT TTCACGGTTA CTGTTCTGA
AAAGAAAAAT CCACCTAGCC CTATCAAGTC AAAGATTCCC CTTTCTGCAC CTCGGAAAAA
TACCAACTCA GTGAAATACA GACTCAAGTT TCGCTTGGTA TAATATTAAT CTTGGCCTTG
TGAGAAACCA TTCTTCCCC TCAGGAGTTT CTATAGGTGT CTTCAGAGTT CTGAAGAAAA
ATTACTGGAC ACAGCTTCAG CTATACTTAC ACTGTATTGA AGTCACGTCA TTTGTTTCAG
TGTGACTGAA ACAAAATGTT TTTTGATAGG AAGGAAACTG (SEQ ID NO: 6)

FIGURE 2

MSLSFLLLLF FSHLILSAWA HGEKRLAPKG QPGPAATDRN PRGSSSRQSS SSAMSSSSAS
SSPAASLGSQ GSGLEQSSFQ WSLGARTGSL YCRVGIGFHL QIYPDGVNG SHEANMLSVL
EIFAVSQGIV GIRGVFSNKF LAMSKKGKLH ASAKFDDCK FRERFQENSY NTYASAIHRT
EKTGREWYVA LNKRGKAKRG CSPRVPQHI STHFLPRFKQ SEQPELSFTV TVPEKKNPPS
PIKSKIPLSA PRKNTNSVKY RLKFRFG (SEQ ID NO: 7)

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FIGURE 3

ATGAGCTTGT CCTTCCTCCT CCTCCTCTTC TTCA GCCACC TGATCCTCAG CGCCTGGGCT
CACGGGGAGA AGCGTCTCGC CCCAAAGGG CAACCCGGAC CCGCTGCCAC TGATAGGAAC
CCTAGAGGCT CCAGCAGCAG ACAGAGCAGC AGTAGCGCTA TGTCTCCTC TTCTGCCTCC
TCCTCCCCCG CAGCTTCTCT GGGCAGCAA GGAAGTGGCT TGGAGCAGAG CAGTTTCCAG
TGGAGCCTCG GGGCGCGGAC CGGCAGCCTC TACTGCAGAG TGGGCATCGG TTTCCATCTG
CAGATCTACC CGGATGGCAA AGTCAATGGA TCCCACGAAG CCAATATGTT AAGTGTTTTG
GAAATAATTG CTGTGTCTCA GGGGATTGTA GGAATACGAG GAGTTTCAG CAACAAATTT
TTAGCGATGT CAAAAAAAGG AAAACTCCAT GCAAGTGCCA AGTTCACAGA TGACTGCAAG
TTCAGGGAGC GTTTCAAGA AAATAGCTAT AATACCTATG CCTCAGCAAT ACATAGAACT
GAAAAAACAG GGCGGGAGTG GTATGTTGCC CTGAATAAA GAGGAAAAGC CAAACGAGGG
TGCAGCCCCC GGGTTAAACC CCAGCATATC TCTACCCATT TTCTTCCAAG ATTCAAGCAG
TCGGAGCAGC CAGAACTTTC TTTCACGGTT ACTGTTCTG AAAAGAAAAA TCCACCTAGC
CCTATCAAGT CAAAGATTCC CCTTTCTGCA CCTCGGAAAA ATACCAACTC AGTGAAATAC
AGACTCAAGT TTCGCTTTGG A (SEQ ID NO: 8)

FIGURE 4

HGEKRLAPKG QPGPAATDRN PRGSSSRQSS SSAMSSSSAS SSPAASLGSQ GSGLEQSSFQ
WSLGARTGSL YCRVGIGFHL QIYPDGVNG SHEANMLSVL EIFAVSQGIV GIRGVFSNKF
LAMSKKGKLH ASAKFTDDCK FRERFQENSY NTYASAIHRT EKTGREWYVA LNKRGKAKRG
CSPRVKPQHI STHFLPRFKQ SEQPELSFTV TVPEKKNPPS PIKSKIPLSA PRKNTNSVKY
RLKFRFG (SEQ ID NO: 9)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 97/02338

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 6 C12N15/12 C07K14/50 C12N15/86 A61K48/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 6 C12N C07K A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF INVESTIGATIVE MEDICINE, vol. 43, no. SUPPL.2, 1995, page 278A XP002034595 GIORDANO F.J. ET AL.: "Reduced ischemia after recombinant Adenovirus mediated in-vivo fibroblast growth factor-5 gene transfer." see abstract	1-4
A	---	5-15
Y	CANCER AND METASTASIS REVIEWS, vol. 9, 1990, pages 191-202, XP002034598 YAYON A. AND KLAGSBURN M.: "Autocrine regulation of cell growth and transformation by basic fibroblast growth factor." see abstract	1-4
A	---	5-15
	-/--	
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input type="checkbox"/> Patent family members are listed in annex.
* Special categories of cited documents :		
*'A' document defining the general state of the art which is not considered to be of particular relevance		
*'E' earlier document but published on or after the international filing date		
*'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
*'O' document referring to an oral disclosure, use, exhibition or other means		
*'P' document published prior to the international filing date but later than the priority date claimed		
*'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention		
*'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone		
*'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art		
*'A' document member of the same patent family		
2	Date of the actual completion of the international search	Date of mailing of the international search report
	8 July 1997	23.07.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer: Mandl, B

INTERNATIONAL SEARCH REPORT

International	Application No
PCT/US 97/02338	

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ONCOGENE, vol. 6, no. 8, 1991, pages 1437-1444, XP002034596 COULIER F. ET AL.: "Putative structure of the FGF6 gene product and role of signal peptide." see the whole document, especially from p.1441, last paragraph to p.1442 first paragraph and discussion ---	1-15
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 8, 1988, pages 3487-3495, XP002034597 ZHAN X. ET AL.: "The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors." cited in the application see the whole document	1-15
A	CIRCULATION, vol. 84, no. 4, suppl., 1991, page II-420 (A1673) XP002034599 BARR E. ET AL.: "Induction of angiogenesis following in vivo gene transfer into myocardium." see abstract -----	1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/02338

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-3, 3A, 5-15 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

After claim 3 and before claim 4, there is obviously another claim which has not got a number: "A method in accordance with claim 2...." This claim was termed 3A. Claims 11-15, obviously refer to this claim 3A (not to claim 4]) and were searched as such.

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.